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Physicochemical Variables Better Explain Changes in Microbial Community Structure and Abundance under Alternate Wetting and Drying Events

Abbas Ali Abid [1,2], Xiang Zou [1,2], Longda Gong [1,2], Antonio Castellano-Hinojosa [3], Muhammad Afzal [2], Hongjie Di [1,2] and Qichun Zhang [1,2,*]

[1] Zhejiang Provincial Key Laboratory of Agricultural Resources and Environment, Key Laboratory of Environment Remediation and Ecological Health, Ministry of Education, Zhejiang University, Hangzhou 310058, China; abbas.ali.abid@gmail.com (A.A.A.); 0621182@zju.edu.cn (X.Z.); 21914103@zju.edu.cn (L.G.); hjdi@zju.edu.cn (H.D.)
[2] College of Environmental and Resource Sciences, Zhejiang University, Hangzhou 310058, China; mafzal.kust@gmail.com
[3] Department of Soil and Water Sciences, Southwest Florida Research and Education Center, Institute of Food and Agricultural Sciences, University of Florida, Immokalee, FL 34142, USA; ach@ugs.es
* Correspondence: qczhang@zju.edu.cn; Tel.: +86-571-8898-2413

Abstract: Soil microbial communities play an important role in nutrient cycling; however, their response under repeated long-term fertilization has attracted little attention and needs further appraisal. A 14-day incubation study compared the relative abundance, diversity, and composition of bacterial and fungal microbial communities in soils treated with long-term applications of chemical fertilizer (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) in a paddy field. A high-throughput sequencing approach was applied to assess the diversity and composition of microbial community. Results revealed the Shannon index of the bacterial community decreased with fertilizer addition but increased in case of fungal community. The abundance of the *Actinobacteria* was higher in the PMCF, while *Proteobacteria* were higher in the CF and SRCF treatments than those in the unamended control under alternate wetting and drying (AWD) and permanent flooding (PF). In addition, chemical fertilizer history increased the abundance of *Firmicutes* under AWD. Initially, *Nitrospira* were found higher in the unamended control than in the amended treatments, but an increase was observed with time in fertilized treatments. Among all genera, *Proteobacteria* were the most abundant bacterial genus. The main properties that markedly affected the bacterial communities were SOC (R^2 = 0.4037, $p < 0.02$), available P (R^2 = 0.3273, $p < 0.05$), and NO_3^- (R^2 = 0.3096, $p < 0.08$). Soil physicochemical factors and biogenic factors explained a variation of 46.27% and 29.35%, respectively. At the same time, 4.59% was the combined effect of physicochemical and biogenic factors. Our results suggested that the physicochemical properties had a more significant impact on bacterial activities than water regime by increasing N and organic matter concentrations in the soils.

Keywords: microbial diversity; wetting; drying; N-fertilization; Hi-throughput sequencing

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1. Introduction

Nitrogen fertilization is an important practice for rice productivity. However, excessive fertilization of the paddy soils results in significant N_2O emissions [1], contributing to about 13–24% of annual emissions of N_2O [2]. About half of the world's population uses rice as a staple food. The annual production of rice is recorded at 480 million tons [3]. In paddy soils, moisture contents and fertilizers can change microbial physiology and activities, directly and indirectly, respectively, by changing soil properties [4]. For example, an increase in moisture content may lead to a shift in the community structure of microorganisms [5].

Soil microbial communities influence the biogeochemical cycling and structure of the soil, and thus play a vital role in the sustainability of the agroecosystem [6–8]. Until now, a plethora of studies have revealed that microbial composition and diversity in different agroecosystems are significantly influenced by manure application. A former study on dry land with a 33-year history of long-term fertilization confirmed that pig manure (PM) significantly promoted alpha diversity and microbial biomass [9]. Additionally, Zhang et al. [10] found that microbial diversity in paddy fields significantly enhanced after PM application. Alternatively, a significant decline in microbial diversity with excessive manure has also been observed [11]. This divergence needs further study in terms of microbial responses to excessive PM application. Additionally, it is more important to investigate the ecology under the application of repeated long-term pig manure and chemical fertilization because repeated fertilization influences soil microbial community by changing the physicochemical and biological properties of the soil [12].

The functionality of a complex and dynamic soil ecosystem depends on the connection of its physical, chemical, biological parameters, and harboring microbes [13]. The balance between maximum microbial activity and diversity explains soil quality [14]. Environment protection can be achieved in a better way by managing soil quality by preserving biodiversity and good agricultural practices [15]. Both crop growth and quality directly or indirectly depend on soil microbial activities, influenced by organic matter (OM) decomposition, mineralization, and nutrient cycling. However, soil quality is not only the subject of biological properties, i.e., the microbial composition of the soil, but physical, chemical, and biochemical properties of soil are also responsible for soil quality [16]. Water-management practices, pore-size distribution, pH, OM, total carbon, nitrogen, microbial enzymatic activity, microbial biomass carbon, and nitrogen also contribute to soil quality [17].

Alternating wetting and drying (AWD) to save water is common practice in many countries such as Japan [18], the Philippines [19], and China [20]. The AWD practice is commonly adopted because of no sacrifice of yield. Zhang et al. [21] revealed that there is no loss in the rice yield compared to continuous flooding (conventional practice) but found this practice a great source of N_2O production [22,23]. Although the effect of AWD on soil microbial community was studied previously, its effect under long-term inorganic and organic fertilization is largely unknown. In addition, no study is available related to the response of microbial communities under different water management and long-term manure and chemical application, especially in eastern China.

The linkage of the effect of manure and AWD on the shift of bacterial and fungal communities is expected to provide a better understating of microbial-mediated processes in paddy soils. Additionally, the pooled outcome of enduring fertilization and AWD on bacterial and fungal microbial structure and diversity is still inconclusive. Moreover, the focus of former long-term studies was on long-term mineral fertilizer rates. Still, the effect of fertilizer types on the soil microbial community structures is largely unknown. Hence, the objectives of the present study were: (1) to compare the effects of long-term organic (PMCF, SRCF) and inorganic (CF) fertilizers on bacterial and fungal microbial community structure and diversity under dry–wet conditions, and (2) to evaluate the most dominant factor influencing bacterial and fungal microbial structure, abundance, and diversity. We hypothesized that "bacterial and fungal soil microbial communities could be changed under long-term fertilization" under an AWD scheme.

2. Materials and Methods

2.1. Soil Sampling Site

The soil samples were collected from a long-term fertilized experiment that was in Jintan county, Jiangsu province of China (120°0′41″ E, 29°57′9″ N) (Figure 1). The study area's annual mean temperature and precipitation are 16.27 °C and 1452.5 mm, respectively. The experiment site had five years of canola-rice rotation history. The four treatments of the soil samples were grouped, with no fertilization (CK/Control), 100% chemical fertilization (CF:N 314 kg ha^{-1}, P 13.77 kg ha^{-1}, K 127 kg ha^{-1}), pig manure compost

plus 50% chemical fertilization (PMCF: Pig manure 6000 kg ha^{-1} + N 157 kg hm^{-2}, P 13.77 kg ha^{-1}, K 127 kg ha^{-1}), and rice straw plus 50% chemical fertilization (SRCF: rice straw 6000 kg ha^{-1} + N 157 kg ha^{-1}, P 13.77 kg ha^{-1}, K 127 kg ha^{-1}). After harvesting rice in 2014, five soil cores from each treatment were collected after the (three replicates). The soil cores were transported to the laboratory after packing in sterile zipped plastic bags and stored at 4 °C. All samples were air-dried after sieving through a 2.0 mm mesh and divided into sub-samples before incubation experiment. Sub-samples were used for soil chemical analysis.

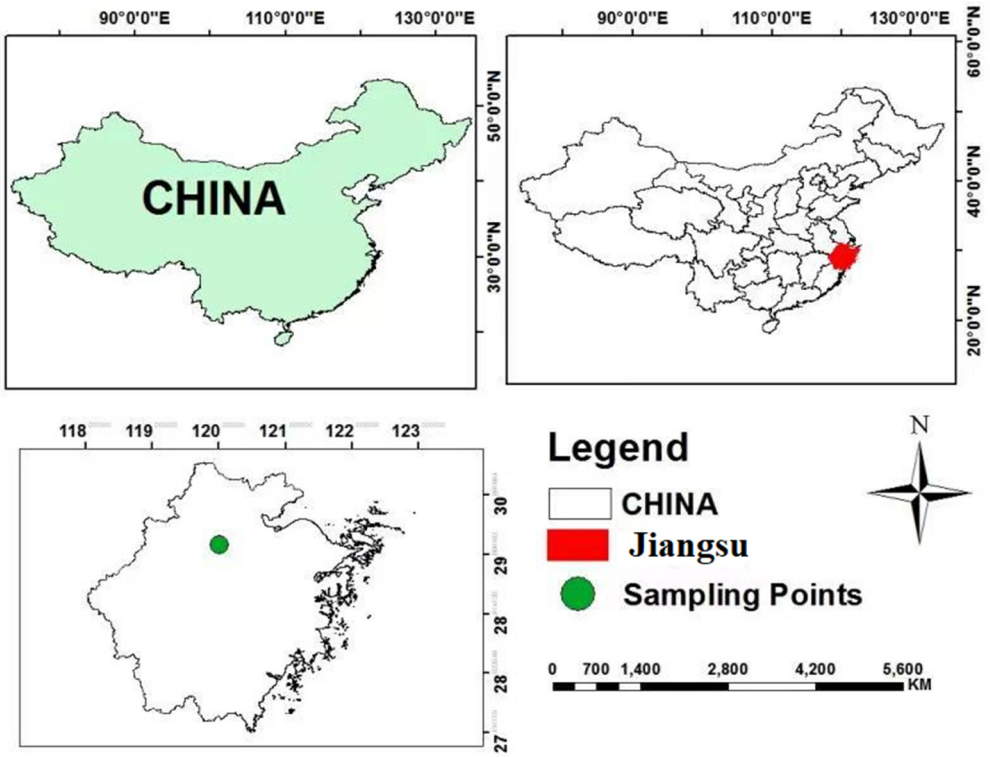

Figure 1. Sample collection point at Jiangsu Province, China.

2.2. Incubation Experimental Design

An incubation experiment was set up for two water regimes: (a) alternate wetting and drying (AWD), consisting of alternating seven days of continuous flooding followed by seven days of air-drying (AWD); (b) permanent flooding for 14 days (PF) at 25 °C. The incubation experiment complete method was described as the 200 g of soil collected from CK, CF, PMCF, and SRCF treatments (with three replicates each) were weighed in 1000 mL glass pots, and flooding conditions were created by adding distilled water up to 3 cm [4,5]. For AWD, flooded incubation was continued for seven days, and gas and soil samples were collected after 1 and 7 days of flooding incubation (100% FC, 1 d; 100% FC, 7 days). Then, the water was removed from pots to reach field capacity of 80% after 10 days (80% FC, 10 d) and 60% field capacity after 14 days (60% FC, 14 days); samples were also collected at 80% FC, 10d, and 60% FC, 14 d. For PF, incubated the soil under flooded conditions by keeping water level 3 cm for 14 d under same fertilizer treatment and soil samples were collected after 1, 7, 10, and 14 d.

2.3. 16S rRNA Gene Sequencing (Illumina-Based)

The DNA of the soil samples was extracted from 0.5 g sampled soil according to the manufacturer's instructions (TAKARA DNA Extraction Kit). The DNA was quantified by Nanodrop technology (NanoDrop Technologies, Wilmington, DE, USA). For bacterial community, V3–V4 region of 16S rRNA gene was amplified using the primers set provided. The bacterial 16S rRNA genes were amplified with the primer 319F (5′-ACTCCTACGGGAGGCAGCAG-

3′) and primer 806R (5′-GGACTACHVGGGTWTCTAAT-3′). The ITS region was targeted with the ITS1F primer (5′-CTTGGTCATTTAGAGGAAGTAA-3′) and ITS2R primer (5′-GCTGCGTTCTTCATCGATGC-3′) [5].

The PCR reaction was performed with an initial melting step for the 30 s at 98 °C, followed by 30 cycles of denaturation at 98 °C for 15 s, annealing at 58 °C for 15 s and elongation at 72 °C for 1 min. After purification, the PCR products were sent to the Illumina Mi-Seq platform for sequencing (www.majorbio.com, accessed on 5 December 2021). Bacterial 16S rRNA gene average length of 435 bp was used for sequencing analysis. The raw data were processed with QIIME 1.8.0 [24]. Sequences were analyzed according to the method described by Edgar [25]. Mi-Seq sequencing analysis was performed using R-Studio 3.5.3, phyloseq 1.26.1 version. High-quality putative bacterial 16S rRNA and fungal gene sequences were confirmed by the BLAST search engine and GenBank database (https://blast.ncbi.nlm.nih.gov/Blast.cgi, accessed on 5 December 2021). The operational taxonomic unit (OTU) was approved with a 97% 16S rRNA gene sequence.

2.4. Statistical Analysis

To examine the significance of treatments, we performed one-way analysis of variance (ANOVA) and Tukey's HSD tests to identify significant differences among the treatments, followed by Duncan's Multiple Range Test (DMRT) to separate treatment means. The vegan package of R studio was used to perform the Mantel test, redundancy analysis (RDA), and variation portioning analysis (VPA). QIIME was used to calculate alpha and beta diversity. Linear discriminant effect size analysis (LEfSe) was performed to recognize the specific bacterial taxa from the phylum to the genus level separately. Bacterial taxa with linear discriminant analysis (LDA) scores of 2.5 or more were considered significant with AWD and PF. All statistical analyses were performed by SPSS 16.0 package (SPSS, Chicago, IL, USA). The results were accepted as significant at 0.05 probability.

3. Results and Discussions

3.1. Soil Chemical Properties

The additions of chemical fertilizer alone and combined with organic amendments significantly decreased soil pH ($p = 0.001$), while this decrease was observed as higher in SRCF treatment (Table 1). Fertilized treatments significantly increased the concentrations of soil organic C ($p < 0.05$), NO_3^--N ($p < 0.01$), and available P ($p < 0.01$), and relatively higher values were observed in the PMCF treatment.

Table 1. Chemical properties of soils collected from long-term experiment plots of the unamended control (Control), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF). Values are mean + standard error of three replicates.

	Treatment	pH	SOC (g kg^{-1})	Total N (g kg^{-1})	NO3$^-$-N (mg kg^{-1})	NH4$^+$-N (mg kg^{-1})	Available K (mg kg^{-1})	Available P (mg kg^{-1})
	Control	6.76 ± 0.08 a	14.02 ± 1.03 a	2.37 ± 0.16 a	11.17 ± 0.65 b	16.94 ± 1.80 b	80.50 ± 12.15 a	9.61 ± 1.47 c
	CF	6.25 ± 0.07 b	18.38 ± 2.17 ab	2.64 ± 0.09 a	20.31 ± 1.73 a	20.27 ± 2.49 ab	69.20 ± 6.69 a	16.98 ± 3.09 ab
	PMCF	6.22 ± 0.04 b	24.63 ± 2.08 a	2.78 ± 0.26 a	19.91 ± 0.29 a	21.08 ± 1.42 a	74.85 ± 8.14 a	22.59 ± 1.31 a
	SRCF	5.54 ± 0.29 c	21.93 ± 2.31 a	2.90 ± 0.29 a	19.80 ± 4.00 a	21.48 ± 2.64 a	86.75 ± 8.94 a	13.04 ± 1.73 bc
		**	*	ns	**	ns	ns	**
F-value		17.76	5.51	1.57	8.13	3.04	0.69	8.44
p-value		0.0007	0.024	0.27	0.008	0.09	0.58	0.007

SOC; soil organic carbon. Values are mean + standard error of the replicates ($n = 3$). The identical letters inside a column show that there is no significant difference 95% probability. Ns—the relationship between treatment and water condition is insignificant. * Significant interaction between treatment and water condition at $p \leq 0.05$ level. ** Highly significant interaction between treatment and water condition at $p \leq 0.01$ level.

3.2. Microbial Biodiversity

The current results showed that fertilizer treatments changed the microbial diversity. Generally, the Shannon and Simpson indices of bacterial community declined with fertilizer addition (Table 2). However, the Shannon indices of the fungal community were promoted

by fertilizer addition, while Simpson indices declined (Table 2). While comparing fertilizer treatments, SRCF promoted Shannon and Simpson indices in the bacterial community. In addition, the Chao 1 index declined in fertilizer treatments. PMCF promoted the Shannon index more during PF than AWD, but not in other treatments. The highest Shannon index was found in PMCF during AWD for the fungal community. For a certain period, the Shannon and Simpson indices of the bacterial community increased and then started to decrease. The goods coverage for the samples was observed as higher than 0.99, which showed that the obtained sequence libraries covered almost all microbial diversity.

Table 2. Changes over time in α-biodiversity indices of nitrifying and denitrifying bacteria and fungi based on 16S rRNA gene in soils collected from long-term experiment plots of the unamended control (Control), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) under alternate wet–dry conditions (AWD) or permanent flooding (PF).

	Treatment	Time (d)	AWD				PF			
			Shannon Index	Simpson Index	Chao Index	Coverage	Shannon Index	Simpson Index	Chao Index	Coverage
Bacterial	CK	1	6.55	0.004	2824	0.98	6.54	0.004	2824	0.98
		7	6.92	0.003	3175	0.97	6.92	0.002	3175	0.97
		10	6.9	0.002	2949	0.97	6.77	0.002	2979	0.97
		14	6.75	0.004	3080	0.97	6.75	0.003	3041	0.96
	CF	1	5.77	0.023	2613	0.98	5.77	0.022	2613	0.98
		7	6.54	0.005	2704	0.97	6.54	0.004	2704	0.97
		10	6.42	0.005	2696	0.97	6.35	0.004	2536	0.97
		14	6.39	0.008	2645	0.97	6.62	0.002	2671	0.96
	PMCF	1	6.23	0.006	2433	0.98	6.22	0.006	2433	0.98
		7	6.61	0.003	2439	0.97	6.6	0.002	2439	0.97
		10	6.53	0.004	2489	0.97	6.69	0.003	2669	0.96
		14	6.12	0.014	2397	0.97	6.35	0.005	2467	0.96
	SRCF	1	6.45	0.007	2807	0.98	6.44	0.007	2807	0.98
		7	6.38	0.012	2995	0.97	6.37	0.012	2995	0.97
		10	6.87	0.002	3002	0.98	6.72	0.003	2923	0.96
		14	6.67	0.004	2928	0.97	6.68	0.003	2716	0.96
			ns	ns	**	ns	ns	ns	*	ns
Fungal	CK	1	2.99	0.15	450	0.99	2.99	0.15	450	0.99
		7	2.63	0.27	431	0.99	2.63	0.27	431	0.99
		10	2.54	0.23	418	0.99	2.11	0.24	275	0.99
		14	2.21	0.27	359	0.99	1.97	0.35	427	0.99
	CF	1	3.3	0.07	443	0.99	3.31	0.07	443	0.99
		7	3.12	0.09	505	0.99	3.12	0.09	505	0.99
		10	3.22	0.09	441	0.99	2.38	0.17	352	0.99
		14	2.4	0.15	383	0.99	2.62	0.13	321	0.99
	PMCF	1	3.75	0.05	476	0.99	3.75	0.05	476	0.99
		7	3.5	0.06	497	0.99	3.51	0.06	497	0.99
		10	3.89	0.05	512	0.99	2.38	0.21	348	0.99
		14	2.83	0.14	396	0.99	3.28	0.07	505	0.99
	SRCF	1	3.75	0.05	508	0.99	3.75	0.05	508	0.99
		7	3.21	0.04	421	0.99	0.05	0.98	94.6	0.99
		10	1.74	0.42	351	0.99	2.37	0.2	388	0.99
		14	2.42	0.16	284	0.99	2.81	0.14	383	0.99
			ns	ns	ns	ns	ns	ns	ns	ns

Ns—the relationship between treatment and water condition is insignificant. * Significant interaction between treatment and water condition at $p \leq 0.05$ level. ** Highly significant interaction between treatment and water condition at $p \leq 0.01$ level.

3.3. Community Structure and Potential Influencing Factors

The bacterial community structure in fertilized treatments was different with respect to time and different water events. The PCoA results showed the bacterial community

structure of PMCF and SRCF was different from CK and CF (Figure 2), which explained 34.22% of the variation during AWD (Figure 2A) and 32.6% during PF (Figure 2B). Additionally, CF was separated from all treatments during AWD, along with the first component (PC1), which explained 18.29% of the variation. These results suggested that the fertilizer treatments and different water conditions significantly affected microbial community structure, especially bacterial community.

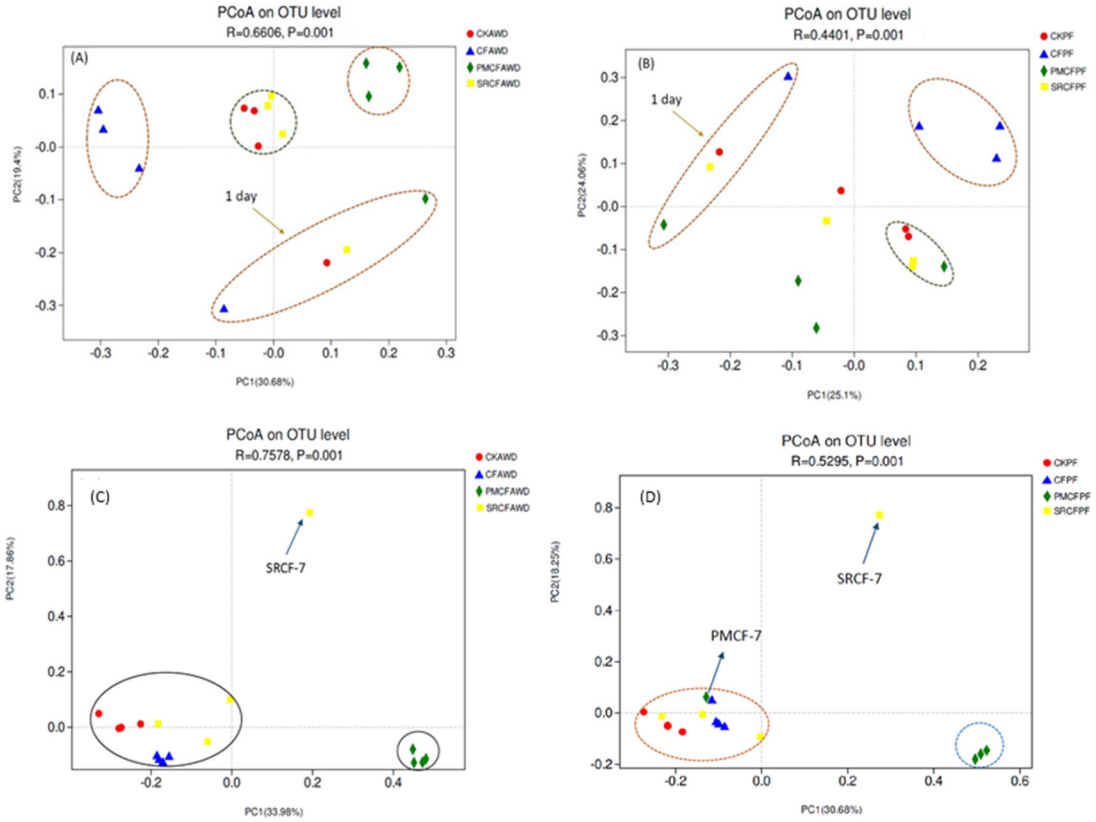

Figure 2. Principal component analysis (PCoA) of bacterial community compositions based on the Bray–Curtis distance for soils collected from long-term experiment plots of the unamended control (CK), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) under alternate wet–dry (AWD) or permanent flooding (PF) conditions at 1, 7, 10, and 14 days after incubation. The PCoA of bacterial community compositions based on the Bray–Curtis distance under AWD and PF is represented by (**A**,**B**), while for fungal community compositions by (**C**,**D**) under AWD and PF, respectively.

The fungal community structure was found to be different from the bacterial community. The PMCF was separated from all treatments along with the first component (PC1), which explained 17.59% variations during AWD (Figure 2C). Meanwhile, SRCF was separated from other fertilizer treatments along with the second component (PC2), which explained the 13.62% variations. The same trends were observed during PF, which showed that moisture contents have no significant effect on fungal community structure (Figure 2D).

The bacterial community was highly diverse compared to the fungal community. For further study of the bacterial community, redundancy analysis (RDA) was conducted to examine the associations between environmental properties (Figure 3). The soil physicochemical properties, including pH, TN, NH_4^+, NO_3^-, SOC, P, and K, explained 57.11% variations during AWD and 55.70% variations during PF. Axis 1 explained 38.79% of the total variability separating CF from PMCF and CK from SRCF-1 (after one-day addition of SRCF) and SRCF-10 (after 10 days of SRCF addition) during AWD. In comparison, Axis 2 explained 17.32% of the total variability, and CK and SRCF were separated from CF and PMCF (Figure 4A). Additionally, during PF, axis 1 explained 44.43% variations and

separated the first day of all treatment applications from later days of all treatment applications, and axis 2 explained 11.27% variations, separating SRCF from all other treatments (Figure 3B). During PF, RDA1 represents TN, NH_4^+, NO_3^-, and SOC of the soils, which were positively related to the bacterial community. At the same time, pH had a negative effect, showing that bacterial community activity is favored by high TN, NH_4^+, NO_3^-, and SOC, and low pH.

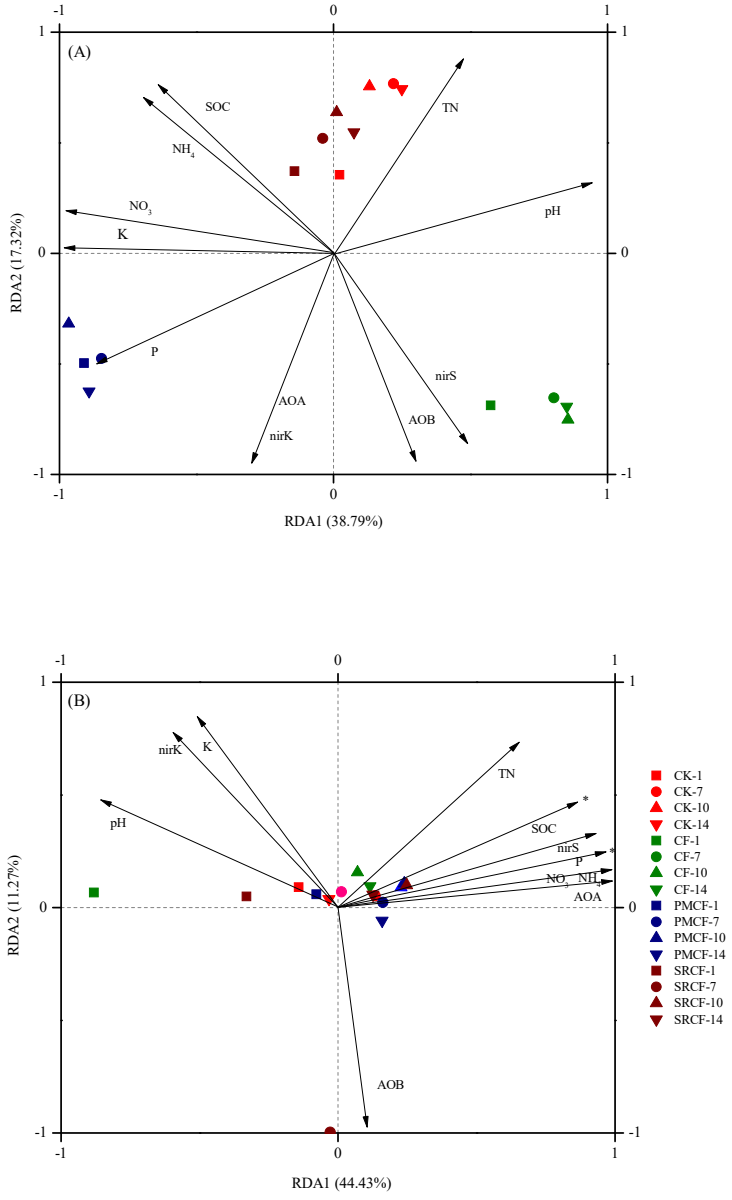

Figure 3. Redundancy analysis (RDA) performed between soil physicochemical characteristics and relative abundance of bacterial communities based on the Bray–Curtis distance at 1, 7, 10, and 14 days after incubation in soils collected from long-term experiment plots of the unamended control (CK), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) under alternate wet–dry (**A**) or permanent flooding (**B**) conditions.

3.4. Species Composition

The overall abundance of the *Actinobacteria* was found to be higher after PMCF addition, while *Proteobacteria* were higher after CF and SRCF additions than those in the CK during AWD and PF (Figure 4A,B). Additionally, *Firmicutes* were higher with CF addition during AWD (Figure 4A). During the early days of the experiment, the *Nitrospira* was

found to be higher in CK treatment but started to increase in fertilized treatments with time. Among all genera, *Proteobacteria* were found to be the most abundant bacterial phylum.

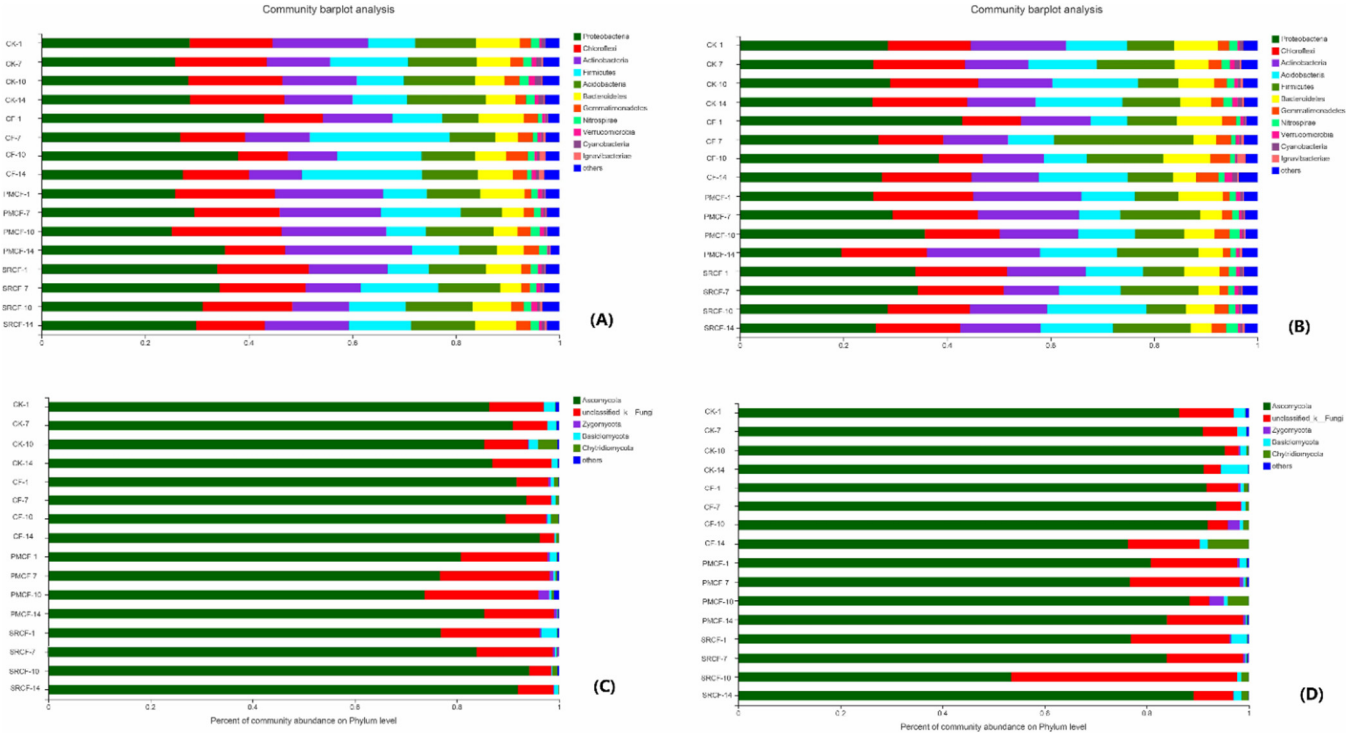

Figure 4. Relative abundance (%) of bacterial community at 1, 7, 10, and 14 days after incubation in soils collected from long-term experiment plots of the unamended control (CK), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) under alternate wet–dry conditions (**A**) or permanent flooding (**B**). Relative abundance of bacterial community under AWD and PF is represented by (**A**,**B**), while for fungal community relative abundance by (**C**,**D**) under AWD and PF, respectively.

The *Ascomycota* was the dominant fungal community in all treatments during both water events (Figure 4C,D). CK promoted *Ascomycota* and *Basidiomycota* compared to the other fertilizer treatments, and *Basidiomycota* increment was five times higher after 14 days of PF compared to AWD. Among fertilizer treatments, CF promoted *Ascomycota* during AWD than PF (20.64% after 14 days), while PMCF inhibited *Ascomycota* abundance until 10 days and then started to improve. CF treatment promoted *Chytridiomycota* during both water events but was four times higher after 14 days of PF (Figure 4D). At the same time, PMCF promoted *Zygomycota* until ten days of fertilizer addition and then started to reduce during AWD. Additionally, SRCF promoted *Ascomycota* by 18.38% and 16.37% after 10 and 14 days of AWD compared to the first day of AWD (Figure 4C).

To explore the effects of WC (AWD and PF), soil physicochemical properties (including pH, available P, available K, TN, SOC, NH_4^+, and NO_3^-), and fertilizers (including CK, CF, PMCF, and SRCF) on bacterial community abundance, a variation partitioning analysis (VPA) was constructed (Figure 5). A Mantel test was conducted to observe the effects of different organic and inorganic sources. The main soil physiochemical properties which markedly affected the communities were SOC (R^2 = 0.4037, $p < 0.02$), available p (R^2 = 0.3273, $p < 0.05$), and NO_3^- (R^2 = 0.3096, $p < 0.08$). A total of 64.46% of the variance could be explained by WC, physicochemical properties, and fertilizer, explaining 3.96%, 29.59%, and 15.52%, respectively (Figure 5). It has also been analyzed that the dominant combined factors which affected the bacterial communities were the interactions between physicochemical properties and fertilizer, explaining 11.84% of the total variance in all the

soils. The physicochemical properties were found to be the dominant factor and affected bacterial community more than water conditions and fertilizers.

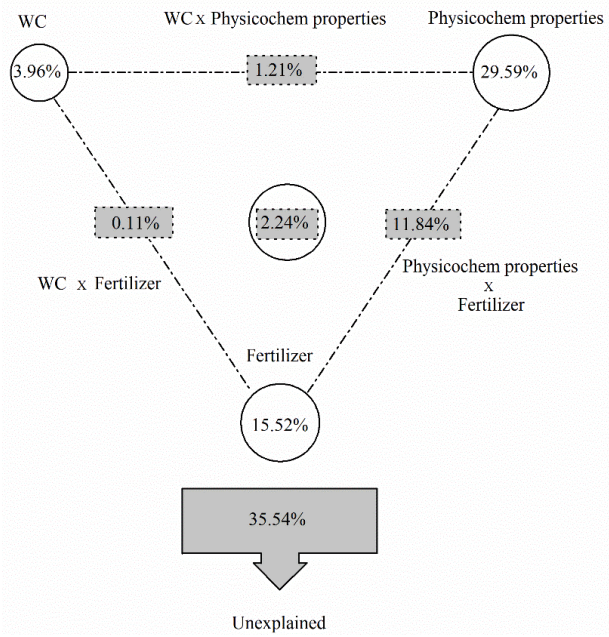

Figure 5. Mantel test and variation partitioning (VPA) analysis of soil physicochemical factors and biogenic factors and the interaction between them and soil water regimes, physicochemical properties, and fertilizer treatments and their interactions. WC—water contents; physicochem properties—physicochemical properties.

3.5. Specific Bacterial Taxa

The LEfSe was constructed to determine the specific bacterial taxa from phylum to genus level separately, affected by the different fertilizer treatments, under AWD and PF (Figures 6 and 7). AWD and PF were separated into two LEfSe figures: microbial populations were grouped as fertilizer treatments and control (Figures 6 and 7). During AWD, we detected 170 clades up to family level from phylum and 28 enriched clades in CF, 83 in the PMCF group, 17 in SRCF, and 42 clades in CK with LDA scores more than 2.5 (Figure 6). The abundance of *Actinobacteria* was found to be higher in PMCF and *Flavobacteria* in CF treatment compared to control, whereas *Acidobacteria* and *Cyanobacteria* remained abundant in CK. Fascinatingly, no significant change was observed at the phylum level in the CK. During PF, only 61 bacterial taxa were identified to have a LDA score higher than 2.5, from phylum up to family level (Figure 7). Only *Actinobacteria* were enriched in PMCF, while *Deltaproteobacteria* were found to be more abundant in CF treatment than in control.

3.6. Long-Term Fertilization and Responses of the Soil Microbiome

The present study showed that long-term fertilization (chemical and organic) application significantly influenced the bacterial and fungal community structures. Bacterial diversity and richness were not significantly changed; however, a decrease in Simpson and Shannon indices of the bacterial community in fertilization treatments was observed. A decrease in fungal diversity was observed with organic fertilizer application, which is highly sensitive to fertilization (Table 2). Previous studies [26–28] of long-term fertilizer application, even for 113 years [29], reported different responses of bacterial and fungal communities, which might be due to the different soil properties and condition. Opposite to our assumption, organic fertilizer applied long term did not change the diversity of the fungal community.

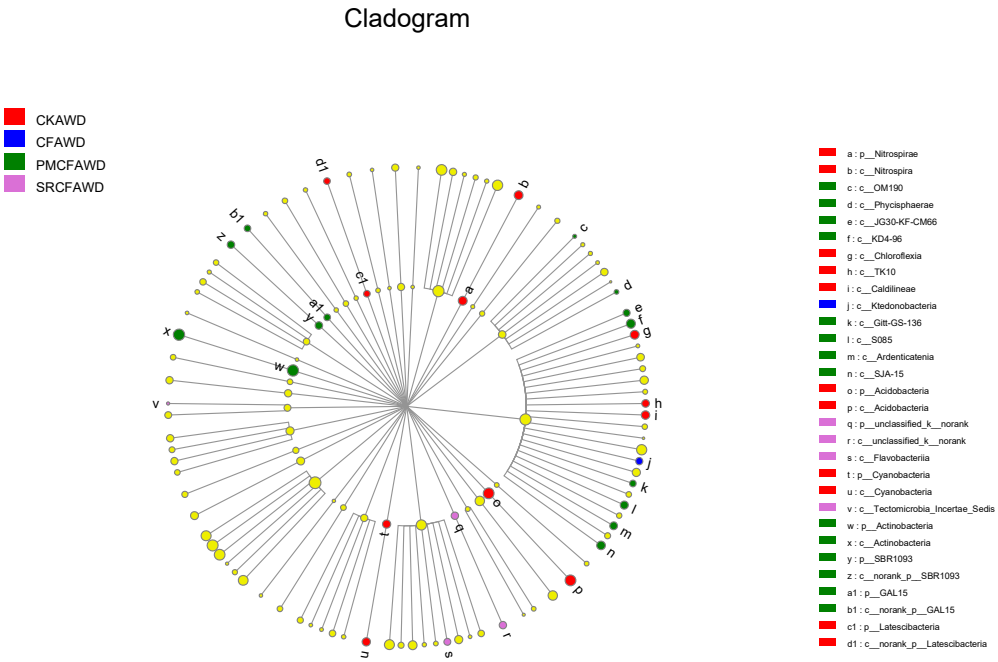

Figure 6. Linear discriminant effect size analysis cladogram of comparison between the unamended control (CK), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) under alternate wet–dry (AWD) conditions. The subsequent colors stand for the abundance of individual taxa. Only taxa meeting a linear discriminant analysis significance threshold of 2.5 were shown. The domain of the cladogram is phylum and class. CKAWD showed CK treatment under AWD condition and so on.

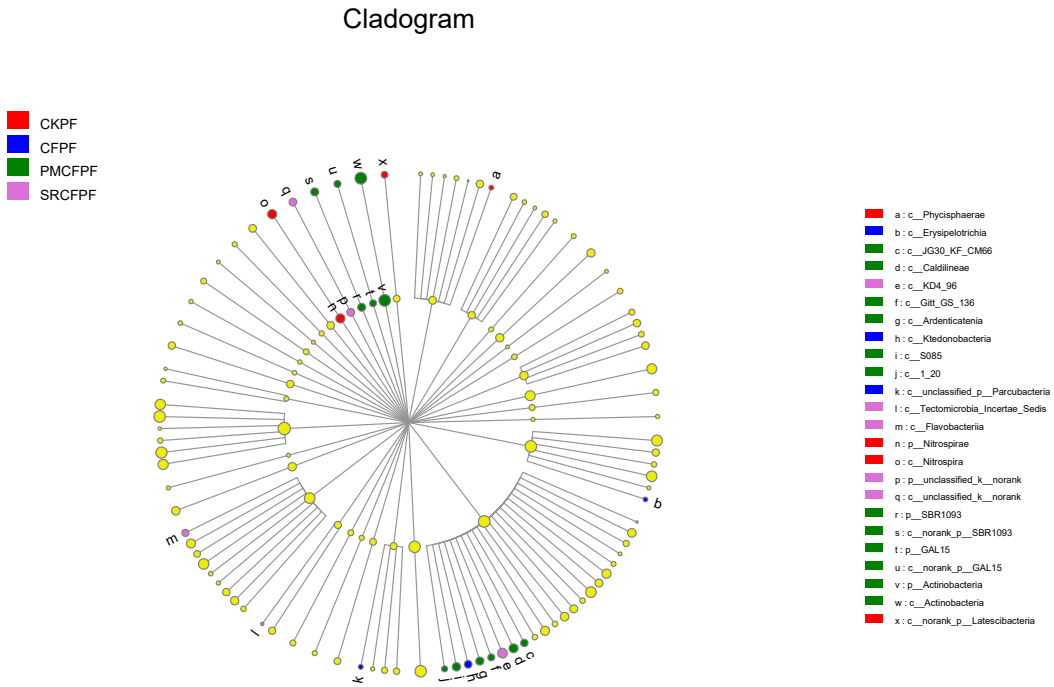

Figure 7. Linear discriminant effect size analysis cladogram of comparison between the unamended control (CK), chemical fertilizers (CF), pig manure plus chemical fertilizer (PMCF), and rice straw plus chemical fertilizer (SRCF) under permanent flooding (PF) conditions. The subsequent colors stand for the abundance of individual taxa. Only taxa meeting a linear discriminant analysis significance threshold of 2.5 were shown. The domain of the cladogram is phylum and class. CKPF showed CK treatment under PF condition and so on.

3.7. Microbial Community Structure under Different Fertilizer Applications and Water Regimes

The current results confirmed that water events and fertilizer treatments changed the relative abundance of the bacterial community, which could further affect the bacterial activities and structure. Some bacterial genera displayed a decrease in relative abundance with different water events and fertilizer treatments [30], which could pertain to this reduction in waterlogging/drought stresses. *Proteobacteria, Bacteroidetes, Chloroflexi, Firmicutes, Actinobacteria,* and *Verrucomicrobia* were abundantly represented phyla during both water events (Figure 4A,B). Of those, *Proteobacteria* and *Firmicutes* were the most abundant Gram-negative bacteria in all fertilizer treatments, consistent with Zhang et al. [30]. Generally, both these phyla members are fast-growing bacteria that prefer a C-rich environment, called copiotrophic bacteria [31,32]. In addition, soil microbial composition might change due to the application of pig manure, which adds external community to the soil, such as *Proteobacteria* and *Firmicutes,* which are common in fecal matter [33]. *Actinobacteria*, an organic matter decomposer, was the dominant phylum in all treatments, especially in NPK treatment, significantly promoting soil quality [34]. The higher level of Gram-negative bacteria was due to the presence of available C. In contrast, Gram-positive bacteria utilize organic C, which needs a more complex set of processes to obtain energy due to its more complex nature [35]. Furthermore, *Nitrospira* was reduced with fertilizer addition because *Nitrospira* is classified as K-strategist that is slow-growing and oligotrophic with a low affinity for N substrates. This result was supported by previous studies of long-term fertilization of 27 years in grasses and eight years in agricultural soils [36].

No significant changes in the fungal community were observed with different water events. *Ascomycota* and *Basidiomycota* were dominant during both water events (Figure 4C,D), consistent with Afzal et al. [5]. These results might be because *Ascomycota* and *Basidiomycota* are the main degraders of the rice straws [6,7]. These results may assume that high and low moisture contents may reduce the fungal community but only favor *Ascomycota* and *Basidiomycota* growth.

RDA results of the bacterial community representing TN, NH_4^+, NO_3^-, and SOC of the soils was positively correlated to the bacterial community (Figure 3). Furthermore, we observed the negative impact of high pH on bacterial growth. However, low pH, an environment rich in SOC, NO_3^-, NH_4^+, and TN, is responsible for promoting bacterial activities. Pig manure and rice straw addition promoted inorganic N and SOC contents; that is why their influence on the bacterial community was high. The input of N either directly influences soil microbiota by altering the inorganic N content of the soil, or indirectly by changing pH, C:N ratio, and SOC availability [37,38]. In addition, soil microbial growth can be enhanced by N fertilizer, which can promote the microbial ability to utilize C sources, and thereby an increase in microbial biomass [39]. A positive correlation between TN and bacteria biomass was found (Figure 3), showing N fertilizer promoted bacterial growth.

In total, 64.46% explained variance, WC, physicochemical properties, and fertilizer explained 3.96%, 29.59%, and 15.52%, variation, respectively (Figure 5). It has also been analyzed that the dominant combined factors affecting the bacterial communities were the interactions between physicochemical properties and fertilizer, explaining 11.84% of the total variance in all the soils. The fertilizer treatments improved the soil's chemical properties and N contents and affected the activities of bacterial communities. Interestingly, physicochemical properties influence the N cycle more than water contents.

4. Conclusions

Verrucomicrobia, Actinobacteria, Firmicutes, Chloroflexi, Bacteroidete, and Proteobacteria were abundantly represented phyla during both water events. No significant changes in the fungal community were observed with different water events. *Ascomycota* and *Basidiomycota* were found dominant during both water events. TN, NH_4^+, NO_3^-, and SOC of the soils were positively correlated to the bacterial community, while pH had a negative effect on the bacterial community. The fertilizer treatments and chemical properties had a more significant impact on the activities of bacteria than moisture conditions. Moreover, in

future studies, underlying mechanisms should be evaluated. Additionally, it is necessary to take more soil biogeochemical processes into consideration in order to better understand the effects of long-term fertilization on the soil.

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